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ANTIBACTERIAL ACTIVITY OF *CINNAMOMUM VERUM* PLANT ESSENTIAL OILS AGAINST MULTI-DRUG RESISTANT *ENTEROCOCCUS FAECALIS* ISOLATED FROM DIARRHEAGENIC CHILDREN

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Abstract

Introduction: Antibiotic resistance is emerging problem worldwide due to different bacterial mechanisms to resist the stress occurred due to antibiotics. This could result into a variety of complications including infections that are challenging to treat with antibiotics, causing longer hospital stays, more severe illnesses and increased mortality rates. Similarly, antibiotic resistance of *E. faecalis* is also increasing everyday leading more enhanced resistance.

Method: In this study, we have isolated the *E. faecalis* from the stool samples of diarrheagenic children followed by biochemical characterization using multiple biochemical tests. However, to further confirm the specie and strain, we have use 16S ribotyping while antibiotic resistance is evaluated by antibiotic sensitivity tests. The plants essential oils are extracted and prepared followed by its agar well diffusion test to determine its efficacy. In addition, Minimum Inhibitory Concentration (MIC) is performed to measure its lowest functional concentration. Furthermore, cytotoxicity analysis of mammalian cells is determined through MTT assay.

Results: The biochemical and molecular characterization confirmed the ten isolates as *E. faecalis* while antibiotic sensitivity test confirms it as Multi-Drug Resistant isolate. The extracted plant

essential oils are evaluated by agar well diffusion tests and found appreciable results of *Cinnamomum verum, Nigella sativa* and *Allium sativum*. However, the MIC results only supported *Cinnamomum verum* for further analysis. Cytotoxicity of all fractions was assessed by MTT and IC50 were 28.28, 14.10 and 28.04mg/mL for n-hexane; n-hexane plus chloroform and ethyl acetate tested by MTT assay, respectively.

Conclusion: It is confirmed that plant essential oil of C. verum is much effective as antibacterial agent with cytoprotective effects on mammalian cells. Therefore, it is recommended to use as antibacterial agent or further evaluation of bioactive compounds.

Keywords: diarrheagenic children, Multi-Drug Resistant, plant essential oils, *Cinnamomum verum*

1. Introduction

Antibiotic resistance is a growing problem that occurs when bacteria develop the ability to resist the effects of antibiotics (Iwu, Korsten, & Okoh, 2020). This can lead to a number of issues including antibiotic-resistant infections can be difficult or impossible to treat, which can lead to longer hospital stays, more severe illnesses, and higher mortality rates (Bouki, Venieri, & Diamadopoulos, 2013). Treating antibiotic-resistant infections is often more expensive than treating infections that can be cured with antibiotics, which can lead to increased healthcare costs (Nair, Tatavilis, Pospíšilová, Kučerová, & Cremers, 2020). Moreover, antibiotic-resistant infections can spread quickly and easily, especially in healthcare settings where patients are often in close proximity to each other (Slayton et al., 2015). This can lead to outbreaks and can make it difficult to control the spread of infections. As more bacteria become resistant to antibiotics, there are fewer treatment options available, which can make it difficult to treat bacterial infections effectively. Furthermore, Overuse and misuse of antibiotics can lead to the development of superbugs which are extremely difficult to treat and can pose a serious threat to public health (Razzaque, 2021). In addition, antibiotic resistance can compromise the immune system, making it more difficult for individuals to fight off infections (Laxminarayan et al., 2013). Concludingly, antibiotic resistance is a serious problem that can lead to difficulty treating infections, increased healthcare costs, the spread of infections, limited treatment options, the development of superbugs, and compromised immune systems (Tanwar, Das, Fatima, & Hameed, 2014). It is important to use antibiotics judiciously to help prevent the development of antibiotic-resistant bacteria.

Enterococcus faecalis is gram positive, spherical shaped, non-motile commensal bacterium which significantly produces lactic acid (Flanagan, 2017). They are generally present in water, soil, fermented food and also habitually in the gastro-intestinal tract (He et al., 2018) but nosocomial infections are the significant feature of the bacteria. They have various routes of transmission via direct person-to-person contact, unhygienic food or environment (Golob et al., 2019). E. faecalis are common cause of bacteremia particularly in immunosuppressive persons and healthcare workers with elevated mortality rate (Noskin, Peterson, & Warren, 1995). Similarly, Enterococci are major cause of wide range of infections including endocarditis, UTI infections, intra- abdominal infections, cellulitis, and wound infections (Noskin et al., 1995). Antibiotics of multiple classes are used for the treatment of the diseases associated with E. faecalis however, drug of choice is selected depending upon the site of infection and antibiotic susceptibility testing. With the passage of time, these bacteria have developed resistance against various antibiotics due to excessive misuse of antibiotics (Rather, Kim, Bajpai, & Park, 2017). E. faecalis and naturally resistant to multiple antibiotics like cephalosporins (Lamb, Figgitt, & Faulds, 1999) while they usually show a potential resistance pattern to multiple antibiotics (Magiorakos et al., 2012). Most of the Enterococci strains are significantly resistant to vancomycin and they transfer the resistance carrying gene to each other (Stępień-Pyśniak, Hauschild, Nowaczek, Marek, & Dec, 2018). The virulence of Enterococci is mainly characterized by various factors such as Enterococcal surface protein (Esp) and aggregation substance (AS) (Raza,

Ullah, Mehmood, & Andleeb, 2018). Ampicillin, gentamicin and vancomycin are the significant antibacterial agents (Golob et al., 2019) before antibacterial resistance but now Vancomycin-resistant enterococci are a major concern in healthcare practices (Celik, Abma, Widdershoven, van Wijmen, & Klinge, 2008). In the enterococci clinical isolates (White, Holleman, Dy, Mirels, & Stevens, 2002), VanA and VanB are reported significantly worldwide (Raza et al., 2018).

The plants extracted compounds can be used as source of drug due to antibiotic resistance ability of bacteria. These compounds include essential oils, volatile compound mixture and aromatic compounds which have antibacterial activity (Negreiros et al., 2016). Essential oils contain phenolic compounds which are much efficient as antibacterial activity (Liu et al., 2020). In case of enterococci infections, non-antibiotic therapeutic options are vaccines, nutraceuticals, immunomodulation agents (Atal, Sharma, Kaul, & Khajuria, 1986), probiotics, phytochemicals and bacteriophages (Clokie, Kropinski, & Lavigne, 2009). Vaccines have been developed to prevent UTI infections to immune the host against infection containing O antigens, fimbriae subunits, α-hemolysin and siderophores. Nutraceuticals have potential to provide health benefits and can be used in medical condition (Clokie et al., 2009). Cranberry (Vaccinium macrocarpon), hyaluronic acid, D-mannose, galabiose, vitamin C, chinese herbal medicine (CHM) and phytochemicals including plants and their secondary metabolic derivatives are the nutraceuticals which are used in case of UTI infections (Loubet et al., 2020).

Essential oils are considered as the most important bioactive substances in medicinal plants. They are also generally volatile compounds (Dorman & (Boyer & Liu, 2004), 2000) and (Dorman & Deans, 2000) extracted from vegetative parts of plant like herb, leaves, twigs, bark, seeds, wood, roots and reproductive parts such as flowers and fruits. Essential oils are obtained by different methods such as fermentation and hydro-distillation (Cassel, Vargas, Martinez, Lorenzo, & Dellacassa, 2009). Due to their hydrophobicity, EOs having the potential to separate with bacterial cell membrane lipids content and results in distortion of bacterial structure (Berman et al., 2002). EOs can be the alternative to the antiseptics and antibiotics and could be effective to the treatment of various infectious diseases (Morse, 2001). Due to emergence of drug- resistant pathogens around the globe, the need of EOs increased for the cure of certain infectious diseases. Antimicrobial activity of EOs against E. faecalis strains was quite significant either in planktonic or biofilm state (Rittmann & Manem, 1992).

C. verum (Cinnamon) member of Lauuraceae family, is native in Sri Lanka, East, and Middle East (Mollazadeh & Hosseinzadeh, 2016). The Cinnamon oil is extracted from bark and leaves by steam distillation (Nuryastuti et al., 2009). Cinnamon oil is effective against cold, flu, arthritis, skin infections and menstrual cramps etc. The taste and odor of cinnamon is because of cinnamaldehyde, other compounds are cinnamyl acid, cinnamyl acetate and eugenol (Kaskatepe, Kiymaci, Simsek, Erol, & Erdem, 2016). *Nigella sativa* known as (black seed), related to Ranunculaceae family, is native in Mediterranean region, Middle East, Asia, and North Africa (Abdallah, 2017). Thymoquinone, linoleic acid, α-pinene, and thymohydroquinone are the main chemicals of *N. sativa*, but thymoquinone has most therapeutic properties (Badri et al., 2018). Essential oils extracted from various plants are effective against the multidrug- resistant Enterococci (Ebani, Nardoni, Bertelloni, Pistelli, & Mancianti, 2018). Immuno-modulant agents (Blasi, Mantero, & Aliberti, 2012) such as Green Tea Extract (GTE) having polyphenolic compounds, especially catechins are effective. Catechins are antioxidants (Shahidi, 2000) and significantly decrease inflammation and uroepithelium edema (Loubet et al., 2020).
The main objective of the study is to characterize the MDR *E. faecalis* from the stool samples of diarrheagenic children and search the most essential oil with improved efficacy to eradicate infection and limited affect to the nearby healthy cells. So, we have used three different essential oils and studied its efficacy against *E. faecalis*

2. Material and Methods

2.1. Sampling, Culturing and Biochemical Characterization

2.1.1. Sampling, Isolation and Identification of *Enterococcus faecalis*

Human pathogenic bacteria *E. faecalis* was isolated by culturing stool samples (n=100) of diarrheic children admitted to children hospital, Lahore. Fecal samples were taken with help of sterile cotton swabs in sterile bags and labelled carefully. Collected stool samples were transported Institute of Microbiology, UVAS, Lahore, at 4°C for further processing. Suspensions were primarily cultured on sterile nutrient agar petri plates and incubated for 24 hours at 37℃. Primary culture was sub-cultured on bile esculin azide agar for the isolation of *E. faecalis*. The preliminary identification of E. faecalis is made by colony characteristics and microscopic identification.

2.1.2. Biochemical Characterization

Gram's positive stained isolates were further processed for biochemical identification using series of tests such as catalase test, oxidase test, indole production test, methyl-red test, Voges Proskaur test and citrate utilization test. Different sugar fermentation was also performed for further confirmation i.e., glucose, mannitol, sucrose, lactose, maltose, salicin, and inosi (Castillo-Rojas et al., 2013).

2.2. Molecular Characterization

2.2.1 DNA Extraction

The overnight culture was used for DNA extraction using GeneAll® Exgene™ DNA Extraction kit. The culture was centrifuged at 14000 rpm for 5 minutes then discard supernatant and pellet was washed with normal saline followed by DNA extraction from this pellet. Working solution of lysozyme (30mg/mL) in GP buffer, 180 uL was added in bacterial Eppendorf tube containing pellet. Mixing by vortex mixture followed by incubation for 30 min at 37℃. To make extraction RNA free, RNAse A (10mg/mL) (thermos scientific) 20 uL was added in above mixture and incubated at room temperature for 20 minutes. Proteinase K (20mg/mL) added in mixture and incubated at 56℃ in water bath for 30 minutes. Tubes were cooled down at room temperature followed by incubation at 70℃ for 30min. After cooling of tubes at room temperature 200 uL absolute chilled ethanol was added in mixture with gentle mixing. Mixture was transferred to the spin columns with collection tubes. Centrifugation was done at 10,000 rpm for 1 minute. Solution in collection tube was discarded and collection tube again attached to the column. BW buffer (absolute ethanol added) 600 uL was added in column and centrifugation was done at 12000 rpm for 1 minute. Collection tube was discarded and new collection tube attached to the column. TW buffer (ethanol added) 700 uL poured in to column and centrifuged at 12000 rpm for 1 minute twice. Attach the column with new sterile 1.5mL eppendorf tube and add 50-150 uL AE buffer for DNA elution. DNA was quantified (ng/mL) through Nano drop spectrophotometry (Thermo scientific 2000) and stored at -40℃.

2.2.2. Polymerase Chain Reaction (PCR)

The amplification of Enterococcus species targeting 16S rDNA gene was carried out by polymerase chain reaction with 25 uL PCR reaction mixture. For 16S rDNA gene amplification specific primers used following the method of Asghar et al (2016) and the primer sequences used were Forward: 8FLP: 5`-AGTTTGATCCTGGCTCAG-3`, Reverse: XB4: 5`-GTGTGTACAAGGCCCGGGAAC-3`.

PCR Taq polymerase master mix 2X (Bio shop) 12.5 uL, forward and reverse 16S primers (100mM stock, 10pmole working (Annex XVI) (Macrogen)) 1 uL each, 8.5 uL nuclease free water (Bio shop) and extracted DNA as template 2 uL used to prepare the PCR reaction mixture in 0.2mL eppendorf. PCR was carried out in thermos-cycler (Kyretac) at 94ºC for 10 minute, 35 cycles were at 94°C/1 minute, 55°C/1minute, 72°C/2 minute and final extension at 72oC/10minutes. PCR products analyzed by Agarose gel electrophoresis using 1.5% agarose gel stained with ethidium bromide (10mg/mL Annex XVII) and DNA visualized in gel documentation system (Cleaver).

2.2.3 Sequencing and Phylogenetic Analysis

The amplicons were sent for sequencing to Advance Biosciences technology (ABI). JUST bio online software was used to clean the sequences for sequence identity and homology study. Sequence alignment was performed using BLAST NCBI. These sequences after analysis submitted to Genbank NCBI and accession numbers were received. For phylogenetic analysis study, sequences in NCBI data base were downloaded with accession numbers. Phylogenetic tree for Enterococcus species was made using 16S rRNA sequences, neighbor joining algorithm, bootstrap as phylogeny method and 2000 bootstrap replications.

2.3 Antibiotic Resistance Profiling

E. faecalis isolates were subjected to check the antibacterial activity pattern by antibiotic sensitivity assay. For antibiotic sensitivity assay, Kirby- Bauer disc diffusion method was used and results were described following the guidelines of Clinical and Laboratory Standards Institute (CLSI).

2.4 Determination of Antibacterial Activity of Essential Oils

2.4.1 Essential Oils Solvent Fractionation

Select two columns of length 55cm and 25mm diameter and washed with detergent and distilled water. Finally rinse it with methanol and left for drying. Activate silica gel (70-230) (MERCK) at 120°C in oven for one hour. Make slurry of 50g silica gel in a beaker using n-Hexane (other solvents for particular fractionation). Place small piece of glass wool using glass rod, at the narrow edge of columns to block leakage. Pour the slurry with help of glass stirrer in columns. Setting of silica gel in columns by tapping of iron stand with iron rode. Left columns for setting of silica gel in it. Essential oil (Verum) 15g measured in glass vial. Load essential oil in to silica slurry added columns. Take 250mL of n-Hexane (other solvents for particular fractionation) in to separating funnel. And pour solvent upon the column and allow the solvent to flow through the column bed (sample added) in drop wise manner. Collect the fractions in conical flask. After running 250mL solvent through column, air dry the all fractions (in particular solvent) on rotary evaporator and collected in glass vial air tight (Dhiman & Kaur, 2019) as depicted in **Table 01**.

Table 1: Plant essential oil solvent fractionation

Sr. No	Fraction No.	Solvent (s)
1	1	n-Hexane
2	2	n-Hexane + chloroform
3	3	Chloroform
4	4	Chloroform + Ethyl acetate
5	5	Ethyl acetate
6	6	Ethyle acetate + Methanol
7	7	Methanol
8	8	Methanol + Acetonitrile
9	9	Acetonitrile

2.4.2 Agar Well Diffusion Test for Essential Oils and Oils Solvent Fractions

Well-diffusion was performed to check antimicrobial activity of agent (Magaldi et al., 2004). Antibacterial activity of plant essential oils and fractions can be determined by well-diffusion assay. Mueller-Hinton agar plates were prepared in bio-safety cabinet and 6mm diameter wells were cut by sterile well borer. 0.5 MacFarland bacterial suspensions were swabbed on agar plates by cotton swabs. Oils with DMSO (1:1) and fractions with their respective solvents were prepared and 50-70µL volume was pipetted in wells. DMSO and solvents of oil fraction were used in wells as negative control. After the incubation at 37°C for 18-24 hours, plates were observed for zones of inhibition and measured in millimeter (mm(Hassan, Rahman, Deeba, & Mahmud, 2009).

2.5 Minimum Inhibitory Concentration (MIC) of Essential Oils and Oils Solvent Fractions

Minimum inhibitory concentration was performed to evaluate the inhibition of visible growth with minimum amount of antimicrobial as described (Ohikhena, Wintola, & Afolayan, 2017). For this purpose, Mueller Hinton broth was prepared according to manufacturer instructions under sterile conditions (Annex VIII). A 96 flat well micro titration plate was taken and 100 µL of Mueller Hinton broth was pipetted in from 1 to 12th well then 2-fold serial dilution of 100 µL of oil was made from 1 to 10th well.

Bacterial suspension 100 µL was poured in from 1 to 11th well, in 12th well nutrient broth was added and optical density (O.D) was noted at 630nm at 0 time then incubation was done at 37°C for 24 hours. After 24 hours results were noted macroscopically and again O.D was measure at 630nm using spectrophotometer and results were calculated.

2.6 Cytotoxicity Testing of Essential Oils Solvent Fractions

Cytotoxicity of essential oils fractions were performed following the method of (Ohikhena et al., 2017) with minor modifications. Baby hamster kidney 21 (BHK 21) cell line was used for evaluation of cytotoxicity. BHK21 cell line was grown in sterile GMEM (MERCK) (Annex XIX) medium containing 10% Fetal Calf Serum (FCS) in 96 flat well micro-titration plate (SPL). Each well of micro-titration plate was inoculated with 1x105 cells per 300 µL. After growth and 80-90% confluency, cell monolayer washed 2 times with sterile PBS (100 µL in each well). And optical density was taken at 570nm. Two-fold serial dilutions of oil fractions were prepared in sterile GMEM (2-5% Fetal Calf Serum (FCS). Each dilution, 100uL was added on micro-titration plate containing cell growth from well 1st-10th. In 11th well solvents of fractions were added as negative control and 12 well was kept as live cell untreated control. After 24 hours of incubation under 5% CO2 at 37°C, cells were washed with PBS sterile solution.

Cells were stained with 1% crystal violet solution (Almutary & Sanderson, 2016) mixed in equal volume of 3% formalin.. Staining solution 80 µL added in each well and incubated for 30 to 60 minutes at room temperature. After washing with sterile PBS-stained cell lines were air dried overnight. DMSO 50 µL was added in each well of micro-titration plate and optical density was taken at 570nm by ELISA plate reader (Biobase) (Dutra et al., 2012). Cell survival percentage (CSP) was calculated as:

$$\text{CSP:} \quad \frac{\text{O. D of test} - \text{O. D of negative control}}{\text{O. D of live cell control} - \text{O. D of negative control}} \times 100$$

From cell survival percentage EC50 of essential oils fractions was calculated.

Results

3.1 Sampling, Culturing and Biochemical Characterization

All of the stool samples (n=100) primarily cultured on blood agar revealed mixed bacterial growth post 24 hours incubation at 37ºC. 81 Individual bacterial colonies having different macroscopic characters were further cultured on nutrient agar using streak plate method. Pure cultures with gram positive cocci arranged in short chains microscopic morphology were selected for biochemical testing and 33 were positive out of 81 pure cultures. Different biochemical reactions used for genus identification were catalase test, oxidase test, indole production, methyl red,Voges proskauer, citrate utilization, urea hydrolysis and sugar fermentation tests. Bacterial isolates with biochemical profile presented in **table. 2** and 14 were positive for *E. faecalis* out of 33 tested isolates.

Table 2. Culture characters, microscopic morphology and biochemical profileof *Enterococcus faecalis* isolates

S. No.	Type of parameter	Interpretation	*E. Faecalis*
01	Primary culture	Mixed growth	Mixed colonies ofbacteria
02	Pure culture	Pure uniform growth of different colony characters	White, smooth convex colonies
03	Gram staining	Cocci/bacilli/coccobacilli Gram positive or negative	Gram positive cocci in short chains
04	Blood agar	Hemolytic/non-hemolytic	Non-hemolytic
05	Catalase test	Bubble formation= positiveNo bubbles= negative	Negative
06	Oxidase test	Colored=positive,Nocolor=Negative	Negative
07	Indole production test	Pink color= positive Yellow color= negative	Negative
08	Methyl red test	Pink= positive, Yellow= negative	Positive
09	Voges Prauskers test	Precipitate= positive No precipitate= negative	Positive
10	Citrate utilization test	Blue color=positive, Green=Negative	Negative
11	Urea hydrolysis test	Hydrolysis zone= positive No zone= negative	Negative
12	TSI culture	H_2S production= positive No production= negative	Negative
13	Glucose	Fermentation= positive No fermentation= negative	Positive
14	Lactose	Fermentation= positive No fermentation= negative	Positive
15	Sucrose	Fermentation= positive No fermentation= negative	Positive
16	Mannitol	Fermentation= positive No fermentation= negative	Positive
17	Maltose	Fermentation= positive No fermentation= negative	Positive
18	Salicin	Fermentation= positive No fermentation= negative	Positive
19	Inositol	Fermentation= positive No fermentation= negative	Negative
20	Mannose	Fermentation= positive No fermentation= negative	Positive
21	Ribose	Fermentation= positive No fermentation= negative	Positive
22	Galactose	Fermentation= positive No fermentation= negative	Positive
23	Fructose	Fermentation= positive No fermentation= negative	Negative

3.2 Molecular Characterization

Biochemically characterized *E. faecalis* isolates (n=14) were confirmed by 16s rRNA ribotyping. Purity of DNA extracted from fresh broth culture of isolates was estimated using nanodrop and visualized by agarose gel electrophoresis using gel doc at 260nm wavelength as depicted in **Fig. 1**. Highest concentration of extracted DNA was 444.5ng/mL Amplification of 16S rRNA gene by PCR using optimized conditions revealed 1500 base pair bands upon electrophoresis as visualized by gel documentation system. Amplified DNA products sequenced by Sanger dideoxysequencing method and original FASTA files were received**.** Each of the FASTA sequence file was checked for sequence alignment by n-Blast post cleaning by just tool infew cases. Sequences were submitted to NCBI for accession numbers and based upon nucleotide homology out of 14 isolates ten were *E. faecalis*. Nucleotide sequences were used to prepare phylogenetic tree for genetic homology with online available isolates data using clustal-w and MegaX soft wares as depicted in **Fig. 2.**

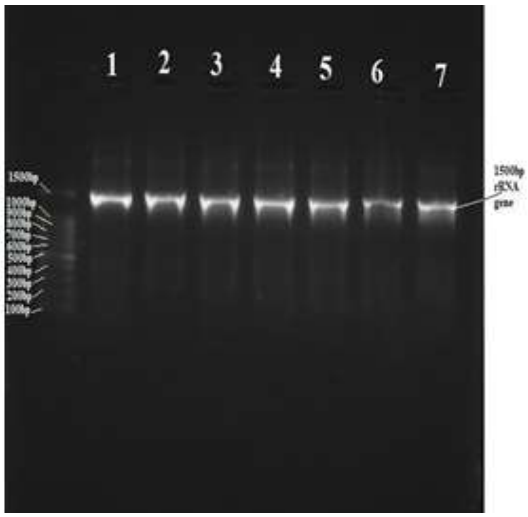

Fig. 1. Whole DNA and 16S rRNA amplified gene electrophoresis pattern in agarose gel

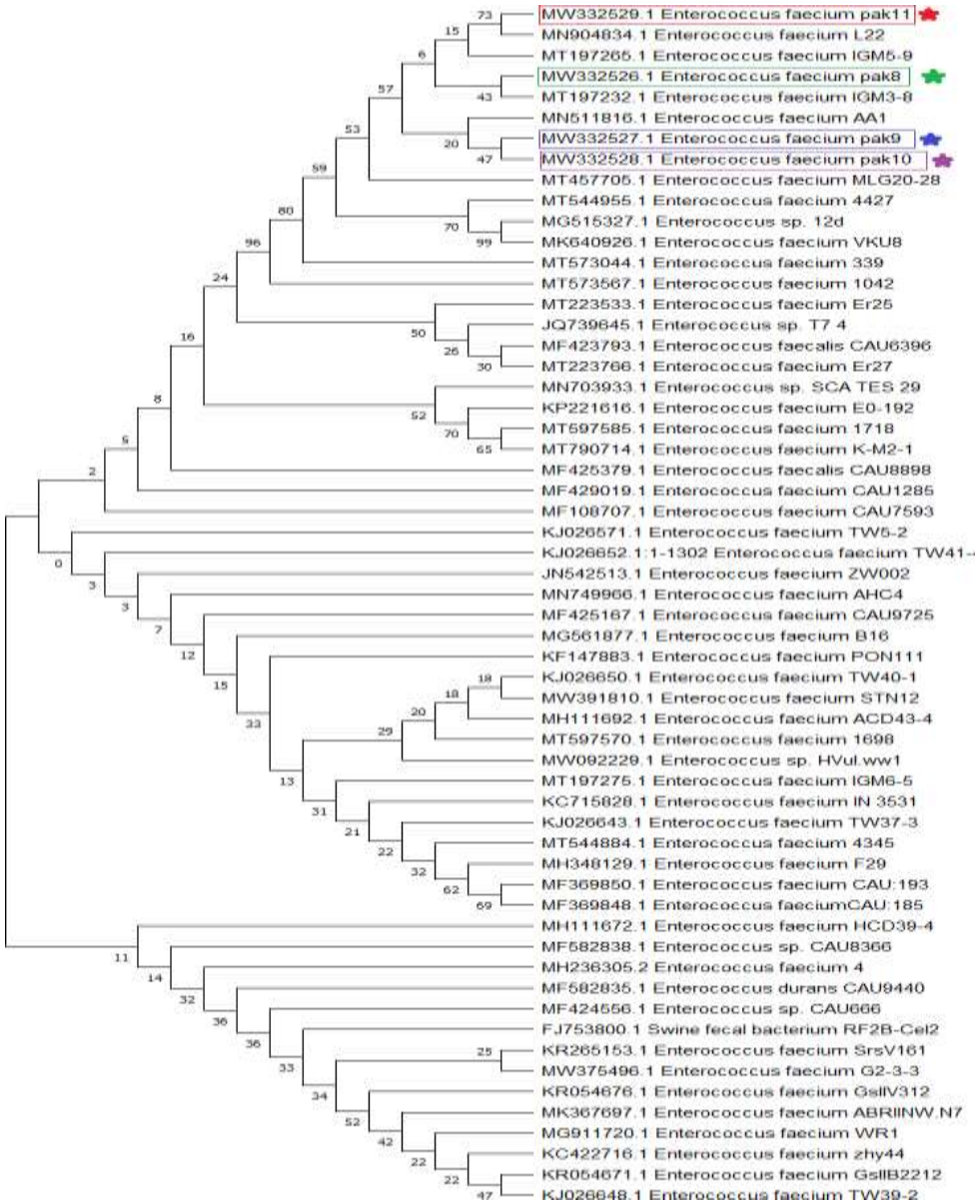

Fig. 2a. It shows the 16S sequencing results of Enterococcus faecalis isolated from the stool of children

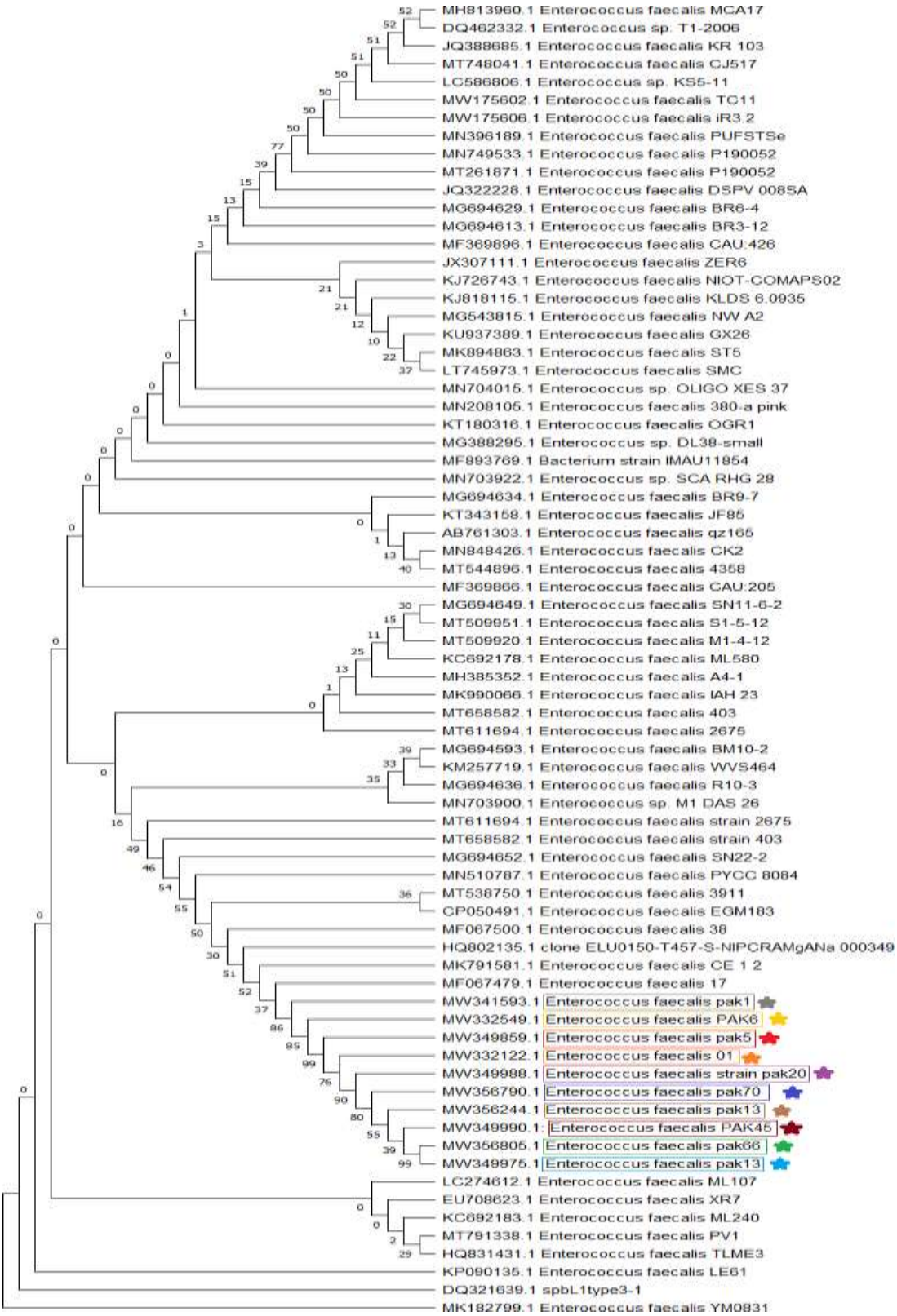

Fig 2b. It shows the 16S sequencing results of Enterococcus faecalis isolated from the stool of children

3.3 Antibiotic Resistance Profiling

E. Faecalis (n=10) were screened for antibiotic resistance to a panel of antibiotic classes. Zone of inhibitions was measured in millimeter (**Table 3**) and compared with standard inhibition zones according to clinical laboratory standard institute (CLSI).

Table 3. Mean zone of inhibitions of antibiotic panel against *Enterococcus Faecalis* isolates

S. No.	Antibiotic class	Name of Antibiotic	Disc Concentration	StandardZOI	Mean testZOI
01	Penicillin	Ampicillin	AMP 10µg	≥ 17	16.00±2.65^{bcd}
		Cloxacillin	CX 30µg	≥ 17	00.00±0.00^{f}
		Oxacillin	OX 1µg	≥ 22	00.00±0.00^{f}
		Amoxicillin	AX 25µg	≥ 18	11.00±1.73^{abcd}
		Piperacillin	TZP 10µg	≥ 21	17.34±2.08^{d}
		Ticarcillin	TI 75µg	≥ 20	00.00±0.00^{f}
		Azlocillin	AZ 75µg	≥ 29	00.00±0.00^{f}
02	Cephalosporin	Cefixim	CFM 5µg	≥ 19	00.00±0.00^{f}
		Ceftriaxone	CRO 30µg	≥ 23	00.00±0.00^{f}
		Cefoxitin	FOX 30µg	≥ 18	00.00±0.00^{f}
		Cefipime	FEP 30µg	≥ 25	00.00±0.00^{f}
		Ceftazidime	CAZ 30µg	≥ 21	00.00±0.00^{f}
		Cephalexin	CL 30µg	≥ 18	00.00±0.00^{f}
		Cefadroxil	CDX 30µg	≥ 18	00.00±0.00^{f}
		Cefuroxime Sodium	CXM 30µg	≥ 18	00.00±0.00^{f}
		Cefamandole	CEF 30µg	≥ 18	00.00±0.00^{f}
03	Carbapenems	Imipenem	IMP 10µg	≥ 19	24.67±7.37^{e}
		Aztreonam	AZT 30µg	≥ 21	00.00±0.00^{f}
		Meropenem	MEM 10µg	≥ 19	00.00±0.00^{f}
04	Macrolides	Erythromycin	E 15µg	≥ 23	00.00±0.00^{f}
		Tylosin	TY 30µg	≥ 15	00.00±0.00^{f}
		Clarithromycin	CLR 15µg	≥ 18	00.00±0.00^{f}
		Azithromycin	AZM 15µg	≥ 23	00.00±0.00^{f}
		Mupirocin	Mu 5µg	≥ 21	00.00±0.00^{f}
05	Tetracycline	Tetracycline	TE 30µg	≥ 15	00.00±0.00^{f}
		Doxycycline	DOX 30µg	≥ 14	9.67±3.21^{abcd}
		Oxytetracycline	OTC 30µg	≥ 15	00.00±0.00^{f}
		Tigecycline	TGC 15µg	≥ 18	16.67±0.58^{cd}
06	Vancomycin	Vancomysin	VA 5µg	≥ 17	9.34±3.06^{abc}
07	Aminoglycosides	Kanamycin	K 30µg	≥ 18	00.00±0.00^{f}
		Enoxacin	EN 10µg	≥ 18	00.00±0.00^{f}
		Spectinomycin	SE 100µg	≥ 15	00.00±0.00^{f}
		Gentamicin	CN 10µg	≥ 15	00.00±0.00^{f}
		Neomycin	N 30µg	≥ 19	00.00±0.00^{f}
		Amikacin	AK 30µg	≥ 17	00.00±0.00^{f}
		Tobramycin	TOB 10µg	≥ 15	00.00±0.00^{f}
		Streptomycin	S 10µg	≥ 15	00.00±0.00^{f}
08	Polymyxin	Colistin	Cl 10µg	≥ 11	00.00±0.00^{f}
09	Chloramphenicol	Chloramphenicol	C 30µg	≥ 18	00.00±0.00^{f}
10	Lincosamide GP	Clindamycin	DA 2µg	≥ 21	00.00±0.00^{f}
11	Folic Acid Synthesis Inhibitor	Sulfamethoxazole	SMZ 100µg	≥ 16	00.00±0.00^{f}
		Trimethoprim	Tr 30µg	≥ 16	00.00±0.00^{f}
		Triple Sulphas	S3 300µg	≥ 19	7.00±4.36^{a}
		Co-Trimazine	Cm 25µg	≥ 18	00.00±0.00^{f}
12	Fusidane	Fusidic Acid	FD 10µg	≥ 19	8.67±7.09^{ab}
13	Fluoro quinolones	Norfloxacin	NOR 10µg	≥ 17	00.00±0.00^{f}
		Ciprofloxacin	CIP 5µg	≥ 21	00.00±0.00^{f}
		Nalidixic Acid	NA 30µg	≥ 19	6.34±3.06^{a}

		Levofloxacin	LEV 5µg	≥ 17	11.34±4.72^{abcd}
		Pefloxacin	PEF 5µg	≥ 24	00.00±0.00^{f}

3.4 Activity of Essential oils against antibiotic resistant *E. faecalis*

Antibacterial activity of plant essential oils (n=10) was checked against three selected *E. faecalis* isolates which were resistant to multiple antibiotics by well diffusion test and zone of inhibitions measured. All of the essential oils exhibited antibacterial activity as depicted from the **Fig. 3.** Highest mean zone of inhibition was observed in case of *Cinnamomum verum* (15.34±0.57) among *Allium sativum, Nigella sativa* and *Cinnamomum verum* as written in **Table 4**.

Table 4. It shows the results of zone of inhibition of different plant essential oils.

S. No.	Name of Essential oils	Test zone of inhibitions againstresistant isolates (mm)			Mean ± S.D. ZOI
		Isolate 01	Isolate 02	Isolate 03	
01	*Nigella sativa*	8	8	12	9.34±2.30^{bcd}
02	*Allium sativum*	7	7	7	7.00±0.00^{abcd}
03	*Cinnamomum verum*	15	16	15	15.34±0.57^{e}

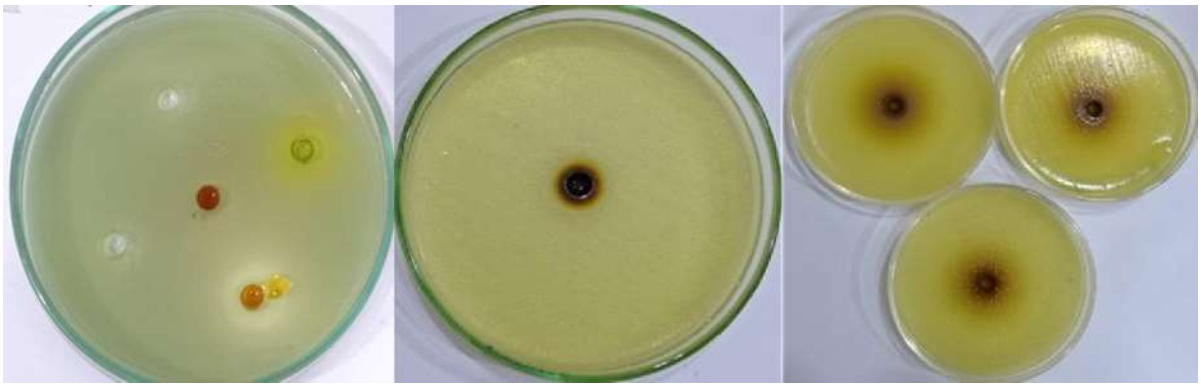

Fig. 3. Activity of essential oils against antibiotic resistant *Enterococcus faecalis*

3.5 Minimum Inhibitory Concentration (MIC) of Essential Oils and Oils Solvent Fractions

The **Table 5.** Shows the results of MIC of various plant essential oils and found appreciable results of C. verum among all plant oils against various strains of *E. faecalis*.

Table 5. It shows the results of MIC of different plant essential oils.

S.No	Name of Essentialoils	MIC values (mg/mL)			Mean MIC ±S.D.
		Iso-01	Iso-02	Iso-03	
01	*Nigella sativa*	55.83	111.67	55.83	74.44±32.24^{b}
02	*Allium sativum*	108.27	108.27	27.07	81.20±46.88^{b}
05	*Cinnamomum verum*	**14.12**	**28.25**	**56.51**	32.96±21.58^{a,b}

3.6 Antibacterial activity of plant essential oil fractions

Four plant essential oils including Cinnamomum verum was selected on the basis of minimum inhibitory concentrations for fractionation by column chromatography. Nine fractions for each of the selected essential oil were obtained using different solvents including n- hexane, n-hexane + chloroform, chloroform, Chloroform + ethyl acetate, Ethyl acetate, Ethyl acetate + methanol, Methanol, Methanol + acetonitrile and Acetonitrile.

The fractions n-hexane and Ethyl acetate differed non-significantly with each other and significantly with rest of the fractions. Mean zone of inhibition of Cinnamomum verum essential oil fractions against resistant Enterococcus Faecalis recorded was the highest for n-hexane (26±8.5) followed by n-hexane plus chloroform (20.00±7.93) and least in case of methanol and Acetonitrile (2.34±4.04).

Table 6: It shows the results of zones of inhibition of different solvent fractions of *C. Verum*

S.No.	Solvent Fractions	Test ZOI (mm)			Mean ZOI ± S.D.
		Iso-01	Iso-02	Iso-03	
01	n-hexane	25	35	18	26 ± 8.5^{d}
02	n-hexane + chloroform	17	29	14	20.00 ± 7.93^{cd}
03	Chloroform	13	25	14	17.34 ± 6.65^{bcd}
04	Chloroform + ethylAcetate	1	11	0	4.00 ± 6.08^{a}
05	Ethyl acetate	7	15	9	10.34 ± 4.16^{abc}
06	Ethyl acetate + methanol	8	7	11	8.67 ± 2.08^{ab}
07	Methanol	0	0	7	2.34 ± 4.04^{a}
08	Methanol + acetonitrile	9	1	9	6.34 ± 4.61^{a}
09	Acetonitrile	7	0	0	2.34 ± 4.04^{a}

3.7 Cytotoxicity analysis of plant essential oil fractions

Cytotoxicity analysis was performed using MTT assay to evaluate the safety of mean MIC values of selected plant essential oil solvent fractions and effective concentration 50 calculated in each fraction was higher than the MIC value. Percent cell survival values of 56.85, 58.28 and 54.60 calculated in case of C. verum selected solvent fractions including n-hexane; n-hexane plus chloroform and ethyl acetate tested by MTT assay were 28.28, 14.10 and 28.04mg/mL, respectively as shown in the graph of **Fig. 4**. The observed concentrations of fractions were equal to or higher than MIC values against tested isolates of *E. faecalis*.

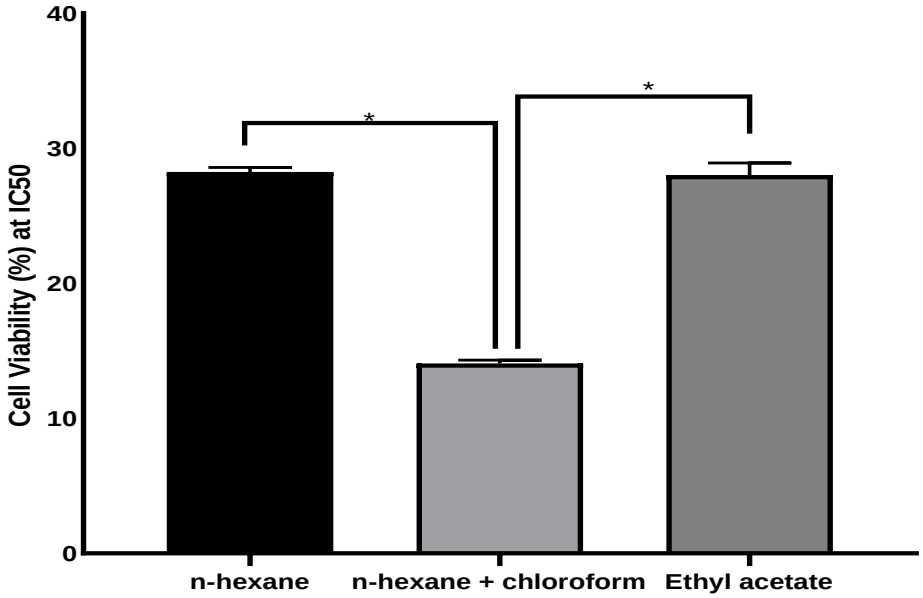

Fig. 4. Cytotoxic analysis of different fractions of C. verum in MTT assay.

4. Discussion

E. faecalis is commonly present in the human and animal intestine (Kim et al., 2016) with main importance in human and veterinary medicine (NI & Huycke, 2014). Due to emergence of antimicrobial resistance *E. faecalis* recognized as nosocomial pathogen of human, especially causing diseases in immunocompromised host (Moura et al., 2013). Humans can contract *E. faecalis* infection through waste water, coastal marine environment, pristine waters, aquacultures, sea foods (Valenzuela, Benomar, Abriouel, Cañamero, & Gálvez, 2010) foals feces (Sukmawinata, Sato, Uemura, & Sueyoshi, 2018) root canals of pulpally-infected teeth (Gajan, Aghazadeh, Abashov, Milani, & Moosavi, 2009) and from stool samples. For isolation of *E. faecalis* BHI, bile esculin agar, Streptococcus selective isolation agar medium was also used (Arumugam, Stalin, & Rebecca, 2017).

On blood agar non-hemolytic small translucent colonies with round edges, black colonies on bile esculin agar and Streptococcus selective isolation agar were observed and it is *E. faecalis* according to(Arumugam et al., 2017). These isolates were biochemically positive for methyl red test, Voges prausker test, glucose, lactose, sucrose, mannitol, maltose and salicin sugar fermentation while negative for catalase and oxidase test. Moreover, these isolates were negative for Indole production, citrate utilization, urea hydrolysis and inositol fermentation. Valenzuela and colleagues reported that *E. faecalis* isolates displayed growth under the effect of 10ºC, 45ºC and NaCl 6.5% broth. Day and colleagues observed similar growth, staining and biochemical characteristics of *E. faecalis* (Day, Sandoe, Cove, & Phillips-Jones, 2001) as observed in present study.

There may be personal error during culturing and biochemical characterization but 16S rRNA gene sequence based genotypic identification is more accurate and reliable method for bacterial identification with defining taxonomic relationships among bacteria (Clarridge III, 2004). By using Ribosomal RNA 16S gene sequence targeted oligonucleotide probe, it was become possible to discriminate the major gut-enterococci specially *E. faecalis* (Harmsen, Prieur, & Jeanthon, 1997). On the basis of 16S rRNA gene sequence analysis followed by NCBI (nblast) in present study bacteria identified as *E. faecalis*. The phylogenetic analysis of the 16S gene of the present 10 *E. faecalis* with the selected 63 published sequences of *E. faecalis* from different species including human discovered the close exclusion in distinctive clade with 16S gene of *E. faecalis* from India. Sequence exclusion evolutionary pattern discovered arbitrary grouping of sequences into different clusters regardless of species of origin of *E. faecalis*. Sequences analysis revealed identity range from 85-100%. Ribosomal RNA 16S sequencing analysis resolve the problem of false negative results for phenotypic and molecular identification (Harwood et al., 2004). On the basis of 16S rRNA sequence several species group (phylogenetic) identified in Enterococcus genus (Williams, Rodrigues, & Collins, 1991). In agreement to present study, the Alsanie and colleagues identified MDR enterococcus on the basis of 16S rRNA sequence analysis with 76-100% identity and phylogenetic tree analysis.

E. faecalis is generally nosocomial and community acquired pathogen and resistance to antibiotics due to intrinsic factors (Moellering Jr, 1992). Scientists proved that clinical isolates have high level of resistance against aminoglycosides, penicillin and vancomycin (Leclercq & Courvalin, 1991). Enterococci act as the reservoir for resistance genes and transfers these genes to human, animals and to environmental bacterial populations (Kim et al., 2016). Generally, antimicrobial resistance pattern in Enterococci depends upon three reasons including: innate resistance against beta lactam and aminoglycosides, resistance acquired from transposable elements plus plasmids and horizontal resistance gene transfer. *E. faecalis* are naturally resistant to cephalopsporins, aminoglycosides, macrolides, sulphonamides, clindamycin and dalfopristin. Enterococci usually show a potential resistance pattern and some strains are multidrug-resistant (MDR). Ampicillin, gentamicin and vancomycin are the significant antimicrobials for the treatment of MDR enterococcal infections (Golob et al., 2019). Vancomycin-resistant enterococci are a major concern in healthcare practices because of major therapeutic use against the MDR enterococci and VanA and VanB antibiotic resistance factors (Raza et al., 2018). *E. faecalis* isolates showed resistance to cephalosporin's, penicillin group, vancomycin, aminoglycosides, folic acid inhibitors, quinolones, meropenems, macrolides, tetracycline and chloramphenicol in the present study. Vignaroli and team detected 16 *E. feacalis* MDR strains and these resistant to erythromycin, tetracycline, vancomycin (Vignaroli, Zandri, Aquilanti, Pasquaroli, & Biavasco, 2011). Unal and colleagues observed similar results to Vignaroli and team, that 78.4% E. feacalis and isolates were MDR (ÜNAL, AŞKAR, & Yildirim, 2017). Zheng and team observed similar result of antibiotic resistance as in present study, that *E. faecalis* VanB positive isolates displayed resistance to erythromycin, gentamycin, kanamycin, tetracycline and vancomycin (Zheng, Tomita, Inoue, & Ike, 2009).

Plant essential oils (EOs) are volatile compounds and secondary metabolites containing bioactive substances. EOs are extracted from flowers, herbs, bark, seeds, wood, roots, leaves, buds and twigs with different methods. In present research, antibacterial activity of essential oils was determined through well diffusion assay and MIC assay against E. faecalis. Highest mean zone of inhibition was

observed in case of C. verum. Against *E. faecalis,* significant activity was observed of *Thymbra capitate* and *Origanum glandulosum* by performing agar disc diffusion. *Ammoides verticillata*, *Lavandula multifida*, *Lavandula dentate* and *Mentha piperita* oils exhibit moderate activity against the stains of *E. faecalis* (Benbelaïd et al., 2014). Some essentials oils are effective against the vancomycin-resistant enterococci (VRE) such as *Eucalyptus globules*, *Kadsura longipedunculata*, *Sideritis erythrantha*, *Citrus limon*, *Citrus sinensis* and *Citrus bergamia* (Solórzano-Santos & Miranda-Novales, 2012).

Components of EOs are usually derived from terpenes and their oxygenated derivatives and terpenoids. EOs in combination with other antimicrobials can broaden the antimicrobial effectiveness against the infectious diseases (Mulyaningsih, Sporer, Zimmermann, Reichling, & Wink, 2010). In present study essential oil fractions in n-hexane, chloroform, ethyl acetate, methanol, methanol, and Acetonitrile (oil fractionation through column chromatography) were evaluated for antibacterial activity against *E. faecalis* through well diffusion assay and MIC. E. cardamomum n-hexane + chloroform and Ethyl acetate + methanol oil fractions give significant antibacterial activity. C.verum n-hexane and n-hexane + chloroform oil fractions results were promising against E. faecalis. Mulyaningsih and team fractioned the cinnamon oil and found that cinnamon aldehyde is effective against gram positive bacteria as in present study cinnamon fraction give best results against gram positive bacteria.

Essential oils are used against microorganism to treat different infections to use them in vivo, in vitro evaluation of them is necessary. Cytotoxicity assays are performed for this purpose on several types of eukaryotic cell lines. It was deduced that cytopathic effect of essential oils is dependent on concentration. Generally, we found higher percentage cell viability observed at 3.25 mg/mL oil concentration. C. verum essential oil 50% inhibitory concentration IC50 was observed 20µg/mL against Vero cells (Azeredo, Santos, Maia, & Soares, 2014). At 0.125% C. verum essential oil concentration, oral keratinocytes viability reduced to 48% and ≤0.0625% concentration no loss of viability was observed (LeBel et al., 2017). C. verum essential oils solvent fractions cytotoxicity evaluated on BHK 21 cell lines. Cell survival percentage ~50% were observed at EC50 14.10-28.28mg/mL.

5. Conclusion

Antibiotics becomes least effective drugs as the antimicrobial resistance is increasing day by day. So, we have to find out the other ways to treat bacterial infections which are more resistance to antimicrobial drugs. In this study we have find out the efficacy of essential oils extracted from plants against MDR *E. faecalis* isolated from the diarrheagenic children and found appreciable results of *C. verum* among all the essential oils. The antimicrobial resistance of *E. faecalis* was evaluated by sensitivity against various drugs while efficacy of essential oils is determined by agar well diffusion test and Minimum Inhibitory Concentration (MIC). The cytotoxicity of Essential Oils was also evaluated by performing MTT and found less toxicity against mammalian cells. Therefore, any compound of group of compounds must be responsible for antibacterial activity of *C. verum* and this is least cytotoxic.

Author Contribution:

All authors contributed equally.

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